

# The C-terminus of myogenin, but not MyoD, targets upregulation of MEF2C expression

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**Abstract** The myogenic regulatory family of basic helix-loop-helix transcription factors, including MyoD and myogenin, functions cooperatively with the myocyte-specific enhancer binding factor 2 (MEF2) family during skeletal myogenesis. Previously, using aggregated P19 cells, we have shown that myogenin upregulates MEF2C expression while MyoD does not [Ridgeway et al., J. Biol. Chem. 275 (2000) 41–46]. In order to identify the domain of myogenin responsible for activating MEF2C expression, a series of chimeras of MyoD and myogenin were generated. Only chimeras containing the C-terminal region of myogenin were able to activate MEF2C in aggregated P19 cells, suggesting that the C-terminus of myogenin is responsible for the regulation of specific target genes. © 2002 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

**Key words:** Myogenesis; P19 cell; Embryonal carcinoma; MyoD; Myogenin; Myocyte-specific enhancer binding factor 2C

## 1. Introduction

The isolation and identification of the myogenic family of basic helix-loop-helix (bHLH) transcription factors (MRFs) represented a major breakthrough in the field of myogenesis [1]. The four MRFs, MyoD, myf-5, myogenin, and MRF-4, bind E box consensus sites found in the promoters of many muscle-specific genes, and activate transcription. In addition to E boxes, another muscle-specific regulatory element, the A+T-rich myocyte-specific enhancer binding factor 2 (MEF2) site, was identified [2]. Four proteins, termed MEF2A–D, that bind the MEF2 DNA binding site were isolated in vertebrates. MEF2 factors are thought to regulate myogenesis through the amplification and maintenance of myogenic bHLH gene expression [3–10].

Several studies in tissue culture have shown differences in MRF function, including differences in the ability to activate both exogenous and endogenous promoters [11–18]. These differences may be due to their ability to remodel chromatin, since MyoD, but not myogenin, has been shown to remodel the chromatin of muscle-specific genes in fibroblasts [18].

Each MRF has a distinct pattern of expression in the de-

veloping mouse somite. The first MRF to be detected in the myotome is myf5, followed by myogenin, MRF4, and MyoD [19,20]. Of the MEF2 factors, MEF2C is expressed first at about the same time as myogenin, followed by MEF2B, -A, and -D [21,22]. Insight into the roles of the MRFs has been gained from gene knockout studies in mice. The distinct phenotypes observed with the loss of individual MRFs, as well as combinations of MRFs, have led to a model in which either myf-5 or MyoD is required for the determination of skeletal myoblasts and/or their propagation [23–25]. Furthermore, myogenin plays an essential *in vivo* role in the terminal differentiation of secondary fibers [26,27]. Using embryonic stem (ES) cells lacking myogenin, it was shown that MyoD cannot compensate for the absence of myogenin during ES cell differentiation [28]. In summary, while each MRF binds to the same core E box sequence, the mechanism by which the MRFs activate different target genes is not clear.

P19 is a line of immortal mouse embryonal carcinoma cells with a stable diploid karyotype. The differentiation of these pluripotent stem cells simulates the biochemical and morphological processes that occur during early embryonic development [29]. In tissue culture, aggregation of P19 cells in the absence of activating drug induces the expression of mesoderm markers, but myogenesis does not occur. However, aggregates treated with dimethylsulfoxide (DMSO) differentiate into cardiac and skeletal muscle along with other mesodermal and endodermal cell types [30]. Skeletal muscle does not appear until day 9 following treatment and represents about 5% of the population. Skeletal myogenesis in P19 cells requires MRFs [31] and the overexpression of MRFs can initiate myogenesis following aggregation in the absence of DMSO [32,33].

Using P19 cells expressing either MyoD or myogenin, termed P19[MyoD] and P19[Mgn] cell lines, respectively, we found that MEF2C expression paralleled the expression of myogenin but not MyoD [33]. Since both MRFs induced similar amounts of skeletal muscle, these results suggest that myogenin selectively targets the upregulation of MEF2C expression, while MyoD does not.

The N- and C-terminal domains of the MyoD family members have been shown to play discriminating roles in the transactivation of many muscle-specific genes [11,14,15,34]. Using a protein chimera strategy, these studies interchange the domains surrounding the bHLH DNA binding/dimerization domain in order to transfer transiently expressed enhancer-specific activation from one MRF to another. From these studies, it was determined that both the myogenin N- and C-termini cooperated in the targeted upregulation of a transiently ex-

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**Abbreviations:** MyHC, myosin heavy chain; PGK, phosphoglycerate kinase; DMSO, dimethylsulfoxide

pressed reporter gene attached to a muscle-specific enhancer [11,14,15], while only the N-terminus of MyoD was found to affect a muscle-specific response [11,14,34].

In order to determine which domain of myogenin targets the upregulation of endogenous MEF2C in stably transfected clones we generated a series of MyoD/myogenin chimeric proteins. The results of these findings suggest that the myogenin C-terminal region is responsible for targeting the upregulation of MEF2C in P19 cells.

## 2. Materials and methods

### 2.1. Plasmid constructs

The three regions of MyoD and myogenin (N-terminus, bHLH, and C-terminus) were generated by polymerase chain reaction (PCR) amplification with appropriate primers flanking the desired end points, as shown in Fig. 1. Insertion of the *Hind*III and *Acc*I sites into the PCR fragments of MyoD and myogenin resulted in the mutation of single amino acids at the junction (Fig. 1B). PCR reactions were carried out with the primers shown in Fig. 1C, under standard conditions as follows: 25 cycles of denaturation at 94°C for 45 s, annealing for 1 min at 57°C, and extension for 1 min at 72°C. The fidelity of each PCR was confirmed by DNA sequencing.

All other expression plasmids were designed using the phosphoglycerate kinase (*pgk-1*) promoter to drive the expression of various cDNAs and have been described previously [33].

### 2.2. Cell culture

P19 cells were obtained from the American Type Culture Collection (No. CRL1825) and maintained, transfected, and differentiated as described previously [35–37]. For each calcium phosphate cotransfection a total of 12.5 µg of plasmid was used: 1 µg of PGK-lacZ, 1 µg of PGK-puro, 2.5 µg of B17 [38], and 8 µg of the desired expression plasmid. Stable cell lines were isolated after selection in puromycin. Differentiation was performed by aggregating cells for 4 days in the absence of DMSO, after which cells were plated into tissue culture dishes. RNA was harvested or cells were fixed for immunofluorescence on day 6 of differentiation. Results were reproduced at least twice with at least two cell lines.

### 2.3. Northern blot analysis

Total RNA was isolated on day 6 of differentiation from P19 cell lines and control cells by the lithium chloride/urea extraction method and subsequent Northern blot analysis was performed as described previously [39]. The probes used were a 600 bp *Pst*I fragment of human cardiac  $\alpha$ -actin last exon [40], a 1.8 kb *Eco*RI fragment of mouse MyoD cDNA [41], a 695 bp *Eco*RI/*Pst*I fragment of rat myogenin cDNA [42], and a 1.5 kb *Hind*III/*Xba*I fragment of MEF2C cDNA [43]. All blots were standardized using a 750 bp *Eco*RI fragment of rabbit 18S cDNA.

### 2.4. Immunofluorescence

Cells were fixed in methanol at –20°C for 5 min, rehydrated in phosphate-buffered saline for 15 min at room temperature, and subsequently incubated with antibody. A mouse anti-myosin heavy chain (anti-MyHC) monoclonal antibody (MF20) was used to stain for total muscle myosin, as described previously [32]. Immunofluorescence was visualized with a Zeiss Axioscope microscope, images were captured with a Sony 3CCD color video camera, and processed using Northern Eclipse.

## 3. Results and discussion

Previous studies have shown that MyoD and myogenin exhibited different target gene specificities in aggregated P19 cells [33]. Myogenin upregulated the expression of MEF2C while MyoD did not. To analyze the mechanism of this target gene specificity, we created chimeric proteins containing domains of both MyoD and myogenin. The chimeric proteins were examined for their ability to activate MEF2C expression

and initiate myogenesis with a view to identifying the regions of MyoD and myogenin responsible for their different biochemical activities. These chimeras are described in Figs. 1 and 3.

To examine whether expression of each MyoD/myogenin chimeric construct in P19 cells can induce differentiation into skeletal muscle, P19 and P19[Myo(XXX)] cells (where XXX denotes either D for MyoD domain or G for Mgn domain) were isolated and cell lines expressing high levels of each construct were aggregated for 4 days and fixed on day 6. Cells were examined by immunofluorescence with antibody directed against MyHC (Fig. 2). Bipolar skeletal myocytes were visible in all cultures (Fig. 2B,D,F,H,J), but not in control cultures (data not shown). Therefore, the amino acid changes shown in Fig. 1 did not affect the ability of MyoD or Mgn to induce myogenesis. Furthermore, these MyoD and Mgn protein chimeras were all capable of inducing myogenesis in aggregated P19 cells.

In order to determine which protein chimeras could activate MEF2C expression, total RNA was isolated from P19[Myo(DDD)], P19[Myo(GGG)], P19[Myo(DDG)], P19[Myo(GDG)], and P19[Myo(GDD)] cell lines on day 6 of differentiation and subjected to Northern blot analysis. High levels of MyoD or myogenin transcripts were present in all cultures transfected with constructs encoding MyoD or myogenin do-

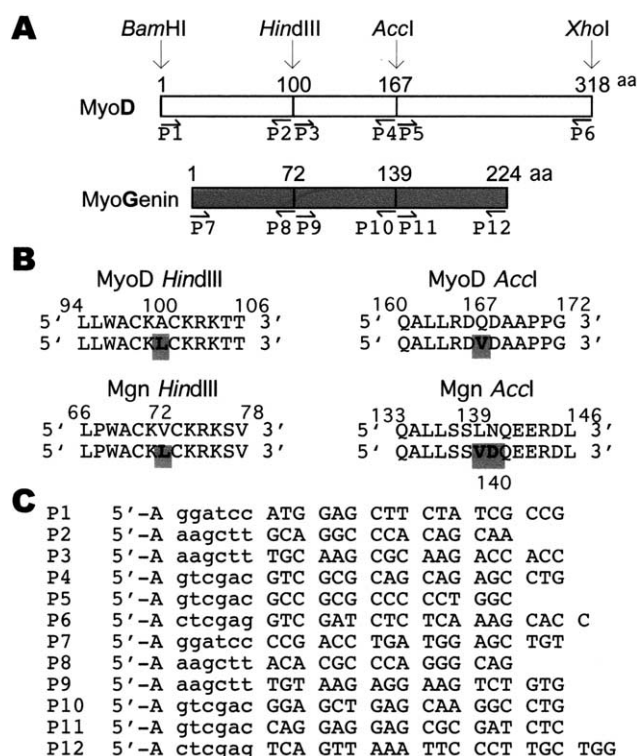


Fig. 1. A: Schematic diagram of the MyoD (white) and myogenin (gray) proteins indicating the sites of mutation and the placement of oligonucleotides for PCR amplification. The positions of oligonucleotides used for amplification of each segment are labeled and the amino acids are numbered. B: Amino acid substitutions required to create restriction enzyme sites in MyoD and myogenin. The mutations A100L and Q167V were made in the MyoD gene to create *Hind*III and *Acc*I sites, respectively. The mutation V72L was made in the myogenin gene to create a *Hind*III site and the mutations L139V and N140D were made to create an *Acc*I site in myogenin. C: Sequences of oligonucleotides (P1–P12) used to amplify each segment of MyoD and myogenin.

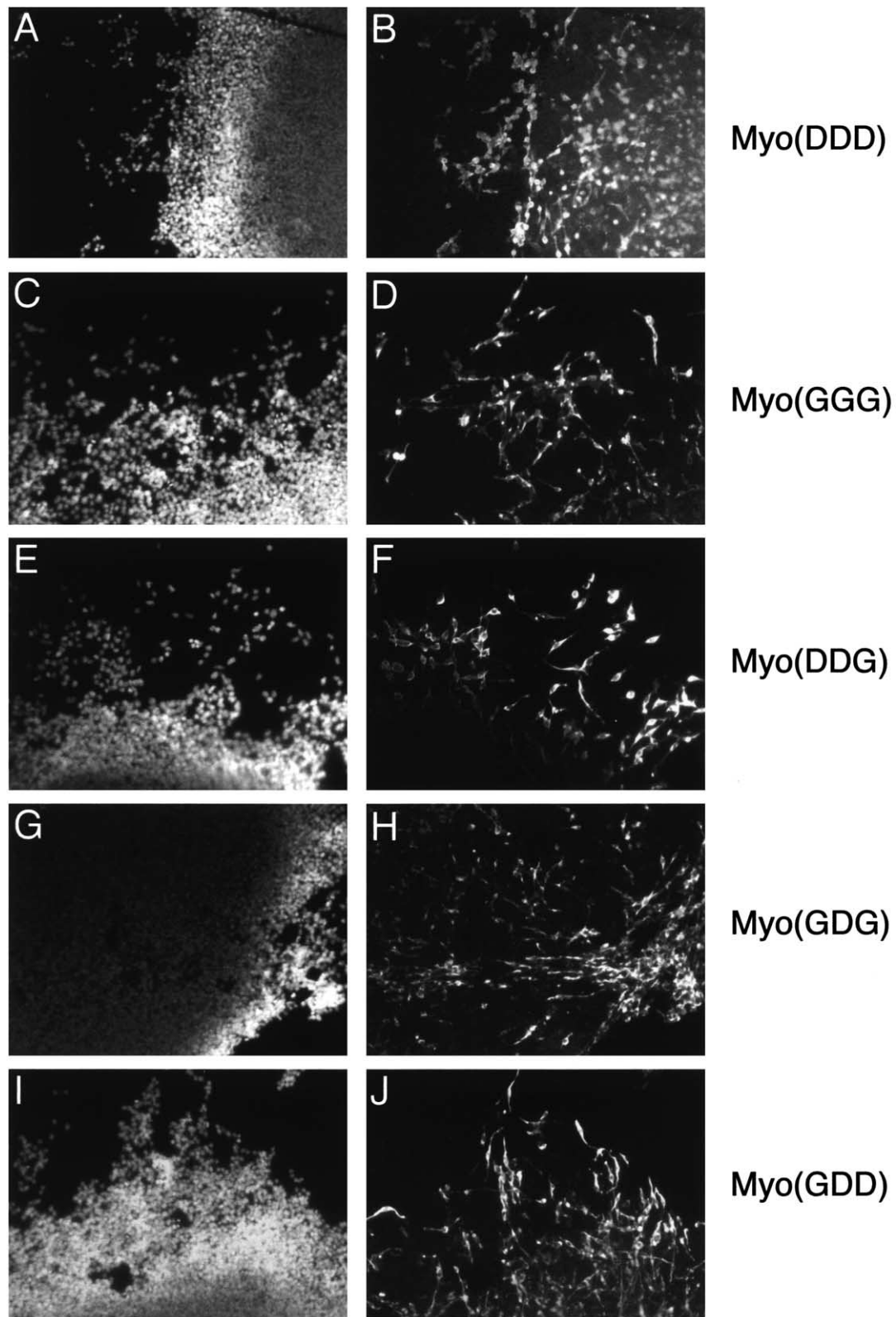
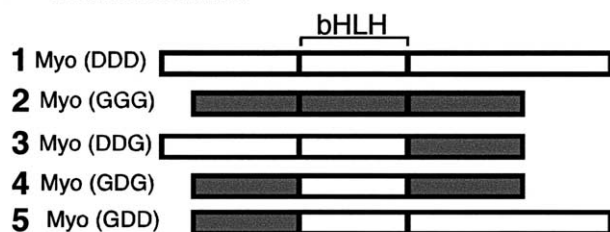


Fig. 2. MyoD/myogenin chimeric proteins induce skeletal muscle development in aggregated P19 cells. P19[Myo(DDD)] (A,B), P19[Myo(GGG)] (C,D), P19[Myo(DDG)] (E,F), P19[Myo(GDG)] (G,H), and P19[Myo(GDD)] (I,J) were aggregated and examined on day 6 by immunofluorescence after reaction with the anti-MyHC antibody, MF20 (observed magnification 80 $\times$ ). Panels on the left show Hoechst staining of the nuclei. Panels on the right show skeletal myocytes identified by MF20 reactivity.

## I. Transfectant



## II. Transfectant

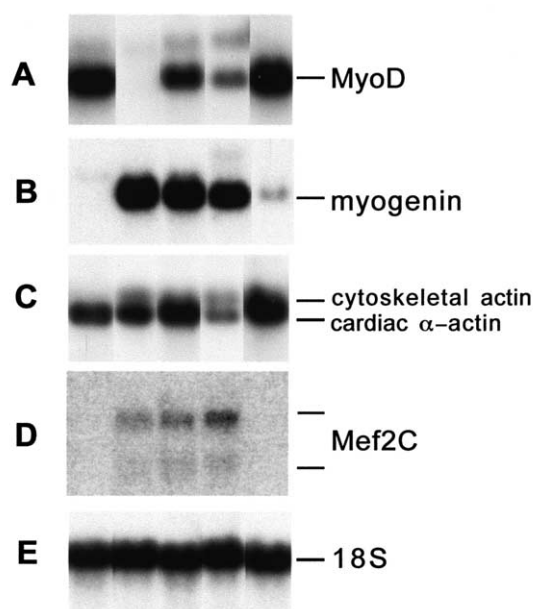


Fig. 3. The C-terminus of myogenin targets upregulation of MEF2C. Total RNA was harvested on day 6 following aggregation from P19[Myo(DDD)], P19[Myo(GGG)], P19[Myo(DDG)], P19[Myo(GDG)], and P19[Myo(GDD)] cell lines. Identical Northern blots containing 6  $\mu$ g of total RNA were probed as indicated on the right. Lanes were spliced from the same autoradiogram.

mainly respectively (Fig. 3II, A,B). The formation of abundant MRF-induced skeletal myocytes is indicated by the high levels of cardiac  $\alpha$ -actin expression present in all cultures (Fig. 3II, C). Myogenin from Myo(GGG) and MyoD from Myo(DDD) did not cross-activate each other's expression to high levels when compared to their exogenous expression levels in differentiated cultures (Fig. 3II, A,B; lanes 1,2), suggesting that the insertion of amino acid changes did not greatly modify the function of MyoD and myogenin [33].

The ability of each clone to predominantly express one MRF or the other allows these cell lines to be used as a tool to study differences in target gene specificity. In agreement with previous results [33], we found that levels of MEF2C expression were upregulated in cell lines expressing myogenin [Myo(GGG)] but not MyoD [Myo(DDD)] (Fig. 3II, D; lanes 1,2). Northern analysis also revealed that cell lines expressing chimeras containing the myogenin C-terminus express comparatively high levels of MEF2C, (clones Myo(GGG), Myo(DDG), and Myo(GDG), Fig. 3II, D; lanes 2–4). Cell lines expressing chimeras lacking the myogenin C-terminus did not express detectable levels of MEF2C (clones Myo(DDD) and Myo(GDD) in Fig. 3II, D; lanes

1,5). This demonstrates a correlation between high levels of expression of chimeric proteins containing the myogenin C-terminus and MEF2C expression.

Attempts to further characterize the region responsible for MEF2C upregulation to a specific subdomain of the myogenin C-terminus were made by swapping subdomains of the myogenin C-terminus into the MyoD C-terminus. Unfortunately, every C-terminal chimera examined upregulated endogenous myogenin expression as well as MEF2C (data not shown). Therefore, these experiments could not distinguish between the ability of C-terminal chimeras to target the upregulation of MEF2C directly by targeting the MEF2C promoter or indirectly by upregulating endogenous myogenin expression. Future studies will require the examination of MyoD C-terminal chimeras in systems lacking endogenous myogenin.

The C-terminal region of myogenin may act to target a specific gene by one or more mechanisms. This domain could function by altering myogenin's ability to bind DNA. Alternatively a change in protein–protein interaction could be used to explain the differences in function, as the myogenin C-terminus contains a known transcriptional activation domain [44]. Finally, it is possible that MEF2C upregulation may be the consequence of a loss of wild-type MyoD structure. This theory would assume that MyoD is normally in a 'repressive' conformation for MEF2C and myogenin expression.

The finding that the C-terminus is responsible for differential targeting of certain muscle-specific promoters is in agreement with previous studies [11,15,34]. In those studies, a domain swapping strategy was used to demonstrate that both myogenin's N- and C-termini cooperated to target the upregulation of a transiently expressed reporter gene attached to a muscle-specific enhancer, while only the N-terminus of MyoD was found to affect a muscle-specific response. As shown previously [45], there are remarkable functional similarities between the N-termini of MyoD and myogenin, as they both contain an acidic activation domain, and cysteine/histidine-rich chromatin remodeling domains.

In summary, while it has been previously shown that MyoD and myogenin exhibit different target gene specificities in aggregated P19 cells [33], the functional domain responsible for this regulation had yet to be identified. The results of our study confirm previous findings that myogenin preferentially upregulates MEF2C [33] in P19 cells. Analysis of chimeric proteins of myogenin and MyoD has revealed that myogenin's specificity for MEF2C upregulation can be localized to its C-terminal domain.

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